PATENT SPECIFICATION

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COMPLETE SPECIFICATION

Antibiotic and its Production

We, BRISTOL LABORATORIES INC., an American Company organised and existing under the laws of the State of New York, United States of America, of P.O. Box 657, Syracuse, New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention is concerned with a new and useful antibiotic, called Amphomycin, and with its production. More particularly, it relates to processes for its production by fermentation, methods for its recovery and concentration from crude solutions including the fermentation broths, purification thereof and the production of salts of its acidic and its basic forms. The invention embraces the antibiotic and its salts in dilute solutions, as crude concentrates and in more purified, solid form.

Reference herein to "Amphomycin" or "the antibiotic" are to be understood to refer either to a single substance or to a group of closely related substances, whose relation to one another is defined by their similar formation and behaviour during the procedures for isolation and purification described below.

During the past few years a number of metabolic products of the growth of bacteria and fungi have been isolated and found to possess valuable therapeutic properties. Among these may be mentioned penicillin, streptomycin, gramicidin, tyrocidin, bacitracin, subtilin, streptothricin, aureomycin and others. Some of these have proven to be extremely valuable because of their effectiveness against pathogenic organisms. Others have been found to be of limited usefulness, as because of their toxicity.

Penicillin is a prominent member of the class of previously described antibiotics which are mainly effective against Gram-positive organisms. Penicillin exhibits several points of weakness, however. Thus penicillin is toxic to certain patients, is relatively inactive orally, is unstable in the presence of water, is inactivated by penicillinase, and tends to lose its effectiveness through development of strains of the organism resistant to the drug.

It is the object of our invention to provide a new antibiotic of good potency, especially against Gram-positive infecting organisms, and suitable for therapeutic use. A further object of the present invention is to provide methods of preparing the above mentioned antibiotic substance which are suitable for commercial use.

There is now discovered, according to the present invention, a process for producing Amphomycin which comprises cultivating a strain of Streptomyces canus in an aqueous, nutrient containing carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to said solution and then recovering the so produced Amphomycin from the fermentation broth.

Within the purview of our invention and as a further embodiment thereof, we have discovered in the process above the steps of decolourizing solutions of Amphomycin by activated charcoal, of extracting the antibiotic into a water-immiscible organic solvent at a pH below 3.5, of precipitating the Amphomycin from aqueous solution by adjusting the pH to a point within the range of pH 3.0 to 4.0, of removing impurities from an aqueous solution having a pH below 3.5 of Amphomycin by extraction of the impurities with methyl isobutyl ketone and amyl acetate, of extracting the Amphomycin from a strongly acid solution in butanol by the use of water having a pH higher than 4, of extracting the Amphomycin from solution in water-immiscible organic solvent into water whose pH is greater than 6.0, of precipitating Amphomycin from solution by formation of insoluble derivatives of the basic function, and of precipitating Amphomycin from solution by formation of insoluble derivatives of the acidic function.

Amphomycin is effective in inhibiting the growth of B. mycoides, B. aureus, M. tetragenus, Staph. aureus and B. subtilis, it forms salts with acids and metals, is soluble in

[Price 3s. 0d.]

Price 3s. 6d.

Price 4s 6d.

Price 75p

water, exhibits minimum solubility in water between pH 3.0 and 4.0, is readily soluble in methanol as the acid form and as the salt form, is soluble in higher alcohols containing at least four carbon atoms only in the acid form, is extractable from water at pH 2 by butanol and pentanol, is not extractable from water at pH 2 by methyl isobutyl ketone, benzene, ether, ethyl acetate and amyl acetate, yields antibacterially active, solid derivatives of ammonium hydroxide and of Reinecke's salt absorbs ultra-violet light only in the 210—230 mu region, exhibits negative response to ninhydrin, Sakaguchi (for arginine and guanidine groups), Molisch, and Ehrlich-Pauly (for histidine and imidazole groups) tests, and is stable for at least ten days in aqueous solution from pH 2 to 10. Details of the Ehrlich-Pauly and Sakaguchi tests are given in the Merck Index, fifth edition, pages 858, 895.

Our novel antibiotic is formed during the cultivation under controlled conditions of a hitherto undescribed species of micro-organism which we have tentatively called Streptomyces canus. The description of this organism is set forth in the following.

The micro-organism canus sp. nov. which produces the new antibiotic substance belongs to the genus currently distinguished as Streptomyces. A mycelium is formed which displays branched hyphæ. Young hyphæ are Gram-positive (older hyphæ variable). Conidia are produced in chains and are spheroidal to ovoidal, measuring 1.0 μ—1.2 μ—by 1.6 μ—1.8 μ.

Growth on glucose-asparagine agar was moderate to good at 30° C. A tan diffusible pigment was produced.

Growth on potato-dextrose agar at 24° C. for two weeks is thin and wrinkled but production of aerial hyphæ and conidia is inhibited.

The organism is characterized by a well branched vegetative mycelium. Numerous, loosely wound spirals are observed when grown at 30° C. on asparagine-meat extract agar (Carbohydrate, 10 gm.; asparagine, 0.5 gm.; beef extract, 2 gm.; K_2HPO_4, 0.5 gm.; agar, 15 gm., and distilled water, 1 litre) containing 1 per cent of either glycerol, sucrose or maltose. The spheroidal spores are produced in chains and measure 1.0 to 1.2 μ in width and 1.6 to 1.8 μ in length.

The following growth characteristics are observed on various media (Waksman, S.A.: The Actinomycetes, Chronica Botanica, Waltham, Mass., 1950) at 30° C. for 14 days.

Potato plug: abundant cream-coloured growth; slight reddish brown darkening of the potato, no aerial mycelium.

Glucose asparagine agar: abundant growth; cream-coloured substratal mycelium turning a russet-brown with aging; abundant slate grey aerial mycelium; heavily sporulated; amber soluble pigment.

Calcium malate agar (Calcium malate, 10 gm.; NH_4Cl, 0.5 gm.; agar, 20 gm., and distilled water, 1 litre): moderate growth; golden substratal mycelium; scant aerial mycelium; no soluble pigment.

Czapek-Dox agar: moderate growth; yellow-brown wrinkled substratal mycelium; scant aerial mycelium; no soluble pigment.

Bennett's agar (Jones, K. L.: Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic, J. Bact. 57:141, 1949): moderate growth; golden wrinkled substratal mycelium; sparse yellow-grey aerial mycelium; faint yellow-brown soluble pigment.

Emerson agar (Baltimore Biological Labs.): abundant growth; amber substratal mycelium; abundant ivory aerial mycelium; heavily sporulated; brownish soluble pigment.

Dextrose nutrient agar; abundant growth; yellow substratal mycelium; white to light yellow aerial mycelium; heavily sporulated; faint yellow soluble pigment.

Potato dextrose agar: moderate wrinkled growth; bronze mycelium; scant aerial hyphae; amber soluble pigment.

The following biochemical reactions are observed when grown on the substrates listed below:

Litmus milk: alkaline with no coagulation; slight peptonization in 14 days.

Gelatin stab: moderate liquefaction at 26° C. in 14 days; no soluble pigment.

Starch: hydrolysis in 96 hours at 30° C.

Nitrate: reduction to nitrite in 96 hours at 30° C. in synthetic medium

Carbon sources: On Pridham's inorganic agar (Pridham, T. G. and Gottlieb, D.: The utilization of carbon compounds by some actinomycetales as an aid for special determination, J. Bact. 36:107, 1948) as the basal medium, growth was observed at 28° C. in 10 days with the following as sole carbon sources: arabinose, rhamnose, xylose, dextrose, galactose, fructose, cellobiose, lactose, maltose, sucrose, dextrin, inulin, raffinose, soluble starch, glycerol, inositol, mannitol, and sodium salicylate. No growth was observed with dulcitol, sorbitol sodium acetate, sodium citrate, sodium formate, sodium malate, sodium oxalate, sodium tartrate, and sodium succinate.

It is to be understood that for the production of Amphomycin we do not wish to limit ourselves to this particular micro-organism as we especially wish to include the use of micro-organisms which are mutants produced from the described micro-organism by mutating agents such as X-radiation, ultra-violet radiation and nitrogen mustards.

Amphomycin shares with penicillin the property of low toxicity and potent activity against bacteria, particularly Gram-positive bacteria. Amphomycin is a valuable therapeutic agent, *e.g.* in human or veterinary

medicine. Amphomycin possesses particular advantages over penicillin by virtue of the fact that it is not inactivated by penicillinase and in certain cases where there are infections due to penicillin resistant strains, where penicillin is not effective and where patients are sensitive to penicillin. Amphomycin exhibits highly useful resistance to degradation by heat or water.

Amphomycin has been found *in vitro* studies to be effective against the Gram-positive bacteria, including, *Staphylococcus aureus, Bacillus aureus, Bacillus subtilis, Bacillus mycoides* and *Micrococcus tetragenus*. The attached chart shows the antibiotic activity of two of the solids prepared from the culture fermentation broth.

PLATE SPECTRUM OF AMPHOMYCIN
(5 mg/nl. of solid)

Organism	Zone of Inhibition in mm. Solid No. 20	Solid No. 17
Bodenheimer Org.	0	0
Proteus X19	0	0
Sh. sonnei	0	0
S. enteritidis	0	0
S. paratyphi A	0	0
S. pullorum	0	0
A. aerogenes	0	0
Ps. fluorescens	0	0
Alc. fecalis	0	0
Pr. vulgaris	0	0
V. cholerae	0	0
Neisseria spp.	0	0
B. mycoides	15	17
B. cereus	15	14
S. marcesscens	0	0
M. tetragenus	14	14
S. flexneri	0	0
S. dystenteriae	0	0
C. albicans $520	0	0
Staph. aureus	14	13
E. typhosa	0	0
E. coli	0	0
S. paratyphi B	0	0
K. pneumoniae	0	0
Ps. aeruginosa	0	0
S. gallinarum	0	0
B. anthracis	0	0
B. subtilis	16	11

The spectrum test is performed as follows: Approximately 30 ml. of sterile heart infusion broth (" Difco " Registered Trade Mark), plus 2% agar to solidify it, is placed in a sterile petri dish (3½" diameter), allowed to harden and then a trench 8 mm. × 40 mm. made in the agar with a sterile spatula. The bottom of the trench is sealed with a drop or two of melted agar. A streak is then made from a 24 hour nutrient broth culture of each test bacteria previously incubated at 37° C., with a small loop, streaking from the edge of the trench to the wall of the petri dish. The trench is then filled with a 5 mg./ml. aqueous solution of the antibiotic. The dish is then placed at 370° C. for 18—24 hours. A linear measurement of the zone of inhibition is then made from the edge of the trench to the point where growth of the test organism exists.

The following is the diffusion plate assay method for determining the activity of Amphomycin:

Culture medium

Streptomycin Assay Agar (with Yeast Extract) was purchased from the Baltimore Biological Laboratories, Baltimore, Maryland and used as directed on the label. A suitable preparation may be made by suspending in one litre of distilled water to a final pH of 8.0 a mixture of 1.5 grams beef extract, 3 grams yeast extract, 6.0 grams peptone (e.g. Gelysate) and 15 grams agar. The suspension is allowed to stand for five minutes, mixed until a uniform suspension is obtained and heated gently with stirring. The suspension is boiled for one or two minutes or until solution has occurred. The culture medium is then dispensed and sterilised at 121° C. (fifteen pounds per square inch of steam pressure, gauge, for fifteen minutes).

Inoculum

The test organism is *Bacillus subtilis* A.T.C.C. 6633. A spore suspension containing 50,000,000 viable spores per ml. is added to melted assay agar (cooled to 53° C.) to give a final inoculum concentration of 2%.

Preparation of Plates

Twenty-one ml. of sterile assay agar are placed in level sterile Petri plates and allowed to solidify. Four ml. of inoculated agar are then distributed evenly over the surface of the base layer. Stainless steel assay plates are placed on the medium after the latter has cooled to room temperature.

Buffer

A phosphate buffer at pH 8.0 is used for making dilutions. This is prepared by mixing 95 ml. of molar K_2HPO_4 with 5 ml. of molar KH_2PO_4 and diluting the mixture to one-tenth concentration with distilled water. The pH of the buffer must be checked potentiometrically and, if necessary, adjusted to pH 8.0 by the addition of one or the other molar phosphate solutions. Variations in pH or concentration of the buffer affect the sizes of inhibition zones markedly. It has not been found necessary to sterilize the buffer. The molar stock solutions are preserved with chloroform and toluene and fresh working solutions are prepared daily.

Assay

Unknown samples are diluted, if required, in the pH 8.0 phosphate buffer. Three depressions on each plate are used to receive a single dilution of the sample. Following incubation at 32° C. the diameter of the zones are measured and averaged.

Amphomycin may be distinguished from Endomycin by the lack of *in vitro* activity

against a strain of the pathogenic fungus, *Trichophyton mentagrophytes*. This is done by placing 25 ml. of a sterile nutrient medium composed of 2% dextrose, 1% neo-peptone, 2% agar, with pH adjusted to 5.6—5.8 in a petri dish and allowing it to harden. An aqueous, spore suspension of *T. Mentagrophytes*, from an agar slent, is spread on the surface, steel penicylinders set on the surface and the plates incubated at 30° C. for 24 hours. An aqueous solution containing 10 mg/ml of the antibiotic to be tested is placed in the cylinders and then the plates returned to incubate at 32° C. for 48 hours. The diameter of inhibition around each cylinder is then measured and an average of at least three zones taken for the reading. The following results were obtained.

Antibiotic	Zone of Inhibition in mm.
Amphomycin—Lot 5	none
Endomycin	24.0
Amphomycin—Lot 1	none

This invention embraces a process for growing a new and hitherto undescribed spacies of micro-organisms, S. canus, at about 24—30° C. under submerged conditions of agitation and aeration on media consisting of a source of carbon, a source of nitrogen, a source of growth substances, mineral salts such as sodium chloride, potassium phosphate, magnesium sulphate, sodium nitrate and, when desired, a buffering agent such as calcium carbonate.

As a source of carbon in the nutrient medium there may be used:

Ordinary starch	Xylose
soluble starch	arabinose
sucrose	rhamnose
glucose	fructose
maltose	lactose
dextrose	inulin
glycerol	dextrins
galactose	

These carbon sources may be supplied to the medium in purified form or in the form of concentrates. The amount of such carbon sources for best antibiotic production in the medium may vary considerably, from about ½% to 5% by weight of the total weight of the fermentation medium.

Suitable sources of nitrogen, including some sources of growth substances, for the fermentation process include a wire variety of substances such as:

Amino acids
casein, both hydrolysed and unhydrolysed
fish meal
soybean meal
meat extracts
liver cake (i.e. liver from which vitamins have been extracted)
urea
nitrates
ammonium compounds
distillers grain slope
corn-steeping liquor
wheat-steeping liquor
whey or whey concentrates
acid hydrolysed corn gluten
acid hydrolysed wheat gluten
peptone
offals
brewers yeast
cottonseed meal
lactalbumin
tryptone.

These proteinaceous ingredients need not be supplied in a high degree of purity; the less pure materials which carry traces of growth factors and considerable quantities of mineral nutrients are suitable for use. It is not possible, of course, because of the crude nature of many of these nitrogenous substances to specify definite proportions of the material to be added. An amount of about 0.1% to 5.0% by weight on a solid basis describes the useful range of nitrogenous substances to be added to the media in most cases.

The pH of the fermentation medium should be 7.0—7.2 at the start of the fermentation. The preferred temperature of the fermentation process is about 26—28° C. The maximum yield of product is usually obtained within 2—7 days, varying with the method of cultivating the Streptomyces.

After growth has been completed, the mycelium is separated from the broth now containing the antibiotic Amphomycin and the antibiotic Amphomycin is recovered from the broth by extraction with organic solvents, by precipitation at the isoelectric point or by other methods as described in this application. The new antibiotic, Amphomycin, produced as afore said, possesses unique and valuable properties which distinguish it from all known and previously described antibiotics.

As is the case with penicillin, Amphomycin is active in vivo as well as in vitro and displays marked chemotherapeutic activity against experimental infection in mice. The results of such testing and of determinations of toxicity are given in the following table.

RESULTS OF IN VIVO TESTING

Amphomycin Lot Number	Response to Plate Assay in mm. (No dil'n)	(1:4 dil'n)	Acute LD_{50} mice i.v. mgms/kg.	CD_{50} mouse i.p. mgm/kg.	Chronic toxicity in mice for 5 weeks mgm/kg.
7	26.0	23.1	—	1.8	>150
8	28.9	27.0	—	0.68	>150
17			>215	2.2	
	(1:16 dil'n)	(1:64 dil'n)			
28	25.0	21.7	46—116	0.57	50—100
Penicillin				about 20	

The CD_{50} (Curative Dose—50) is the minimum intraperitioneal dose of Amphomycin which will cure 50 per cent. of a group of mice injected intraperitioneally with 100 to 1000 LD_{50} doses of *Diplococcus pneumoniae*, each LD_{50} dose being sufficient if given alone to kill 50 per cent. of a group of mice. The infection is given at once after the first dose of the test drug. The test drug is given in two equally divided doses at approximately an 18-hour interval. The animals are observed for four days and deaths for each group expressed as the percentage of the total animals per group. The percentage death is transformed to probit values and these plotted against the log of the dose in mgms per kg. of mouse weight. The point of intersection of the probit line and the best line constructed through the experimental points describes the concentration of drug which should protect half of the animals under the conditions of the experiment. The antilog of this term is called the CD_{50} value.

Amphomycin shows a minimum water solubility in the pH range of 3.0—4.0, and particularly between pH 3.3—3.6, but is very soluble above or below this pH range. It is readily soluble in methanol either as the acid or salt form and in higher alcohols containing at least four carbon atoms in the acid form. It is extracted from water by a butanol or an amylalcohol at pH 2 but not by methyl-*iso*-butyl ketone or amylacetate. From acid butanol solution it may be extracted into water at a pH higher than 3 or 4.

Acid solutions of Amphomycin in butanol may be decolourized with carbon and the acid form of the antibiotic precipitated by addition of non-polar solvents such as ethyl acetate and ether.

An aqueous solution (5—20%) of the acid Amphomycin in water gives a copious precipitate on adjusting the pH to 3.4—3.5 with alkali. The precipitate redissolves as the pH is raised or lowered.

Insoluble derivatives of the basic function have been obtained. Treatment of the sodium salt in water with Reinecke's salt gives no precipitation but lowering the pH to 2—3 gives a good yield of active reineckate salt. The antibiotic is easily regenerated by dissolving the reineckate salt in acetone and adding ammonium hydroxide to precipitate the active ammonium salt of Amphomycin. To date the reineckate has not been crystallised. An oily picrate has been prepared in similar manner.

Amphromycin is stable in water, aqueous solutions at pH 2, 7, or 10 showing no loss after storage at room temperature for 10 days. Solid Amphomycin is stable at room temperature.

The purest material prepared to date shows absorption of ultra-violet light only in the 210—230 mu region and gives negative ninhydrin, Sakaguchi, Molisch, and Ehrlich-Pauly tests. The product contains carbon, hydrogen, oxygen, and nitrogen. The present samples are white or near-white non-crystalline powders. They give a positive biuret reaction and after hydrolysis with 6N HCl for 24 hours at 100° C. the ninhydrin test is also positive. Paper chromatography gives evidence of several different ninhydrin-reacting substances in the hydrolyzate.

The properties of Amphomycin serve to distinguish it from other antibiotics produced by actinomycetes.

Of those actinomycete antibiotics showing acidic properties, antimycin, actinorhodin, and streptomyces antibiotics X—206, X—464 and X—537 (see "Actinomycetes and their Antibiotics" by Waksman and Lechevalier, 1953) are nitrogen-free substances with no basic properties. The free acid of these substances is soluble in non-polar solvent (ether, benzene, etc.), while amphomycin is not.

Litmocidin and rhodomycetin are pigments, while Amphomycin preparations of high potency are white powders.

Of the acidic, nitrogen-containing or amphoteric actinomycetes antibiotics, borrelidin (see "Actinomycetes and their Anti-

biotics" by Waksman and Lechevalier, 1953) is extractable into benzene at acidic pH while Amphomycin is not. Borrelidin has a very limited antibacterial spectrum. The free acid forms of both endomycin and musarin are water-insoluble, while Amphomycin shows a solubility greater than 5% in water at pH 2. Amphomycin is different from aureomycin and terramycin in its lack of UV absorption in the significant 220—400 mu range.

EXAMPLE 1.

PREPARATION OF ACTIVITY IN SHAKER BOTTLES.

To produce small quantities of Amphomycin, the fermentation is conducted in shaker bottles open to the air but protected from contamination with cotton plugs. The Streptomyces canus is grown in a suitable nutrient medium by the submerged culture method, agitation and aeration of the culture mixture effected by placing the bottles on a reciprocating type shaker which provides spraying, splashing or spilling of the mash through an oxygen-containing atmosphere. As a typical case, 500 ml. of a culture medium composed of

1% Soybean Meal
1% "Cerelose" (Registered Trade Mark)
0.5% NaCl
0.05% Curbay BG (a brand of distillers solubles)
0.1% $CaCO_3$

are introduced into 4 litre bottles and sterilised. After autoclaving, the medium is inoculated with about 1%, by volume, of a turbid aqueous spore suspension of the Streptomyces from an agar slant. The pH is 7.0—7.2 at the start of the fermentation. The contents of the bottle are then incubated at 26—28° C. for 120 hours while shaking at 130 strokes per minute with 1¼" stroke. After the incubation period the content contains the following activity given as the diameter (millimeters) of the zone of inhibition at the dilution indicated: (x) 29.2, (4x) 26.2 (determined by the diffusion plate assay procedure described elsewhere). The pH at the end of the fermentation was 7.8.

EXAMPLE 2.

PREPARATION OF PILOT PLANT INOCULUM.

For larger scale production of Amphomycin an inoculum is prepared in a fermentation medium containing, by weight,

1% Soybean Meal
1% "Cerelose" (Registered Trade Mark)
0.5% NaCl
0.05% Curbay BG (a brand of distillers soluble)
0.1% $CaCO_3$

made up in a volume of 2500 ml. and introduced into a 2½ gallon bottle. The medium is sterilised with steam at 118—120° C. for 1 hour. When cool, the medium is inoculated with about 0.5%, by volume, of a turbid, aqueous spore suspension of the Streptomyces canus from an agar slant. The contents of the bottle are then incubated at 26—28° C. for 72—96 hours on a reciprocating type shaker. From the inoculus bottle the broth containing the Streptomyces canus is forced into the tank fermenter under completely aseptic conditions.

EXAMPLE 3.

LARGE-SCALE PRODUCTION OF AMPHOMYCIN.

Amphomycin can be prepared on a large scale by submerged or deep culture of the organism. Stationary vat fermenters equipped with suitable agitation and aeration devices have been found to be useful. A nutrient medium, consisting of 57,000 grams soy bean meal, 57,000 grams "Cerelose" (Registered Trade Mark), 28,000 grams $CaCO_3$ and water to make 1,500 gallons, with a post-sterilisation pH of 6.98, is prepared in a 2,000 gallon glass-lined, steel, vertical vat-type fermenter equipped with a water-jacket for temperature control, stainless steel anchor-type impeller, and a double armed perforated stainless steel plate sparger. The medium is sterilised by heating with steam under pressure and cooled. The nutrient medium is inoculated with 15%, by volume, of a 48 hour vegetative culture grown in a similar type fermenter previously inoculated with an inoculum described in Example 2. The culture in the 2,000 gallon fermented is incubated at 83° F. for 50 hours. During the incubation the impeller is revolved at the rate of 90 R.P.M. and sterile air passed into the medium at the rate of 100 cubic feet per minute. Analysis of a portion of the culture liquid at the end of the incubation period shows the pH to be 7.68 and the activity, determined by the diffusion plate assay described elsewhere, as follows: (x) 18.7; (4x) 14.

The activity is given as the diameter in millimeters of the zone of inhibition at the dilution indicated, that is x indicates undiluted and 4x indicates diluted to four times the original volume.

The isolation of solid Amphomycin from this broth is described in Examples 5 and 6 below.

EXAMPLE 4.

Broth Extraction. Materials are assayed with the use of the cylinder-plate assay response measured in millimeters. Solutions are assayed at several dilution and a comparison made in this way.

A 30 litre batch of filtered broth was acidified to pH 1.95 with HCl and filtered through a pad of diatomaceous filter aid to give a clear solution. The clear filtrate was stirred with 15 litres of *n*-butanol for 15 minutes, and the two

phases separated. The process was repeated with an additional 48 litres of broth and the butanol extracts (40 litres) combined and washed with 10 litres of water acidified to pH 2 with HCl. The butanol was then stirred with 10 litres of water and the mixture adjusted to pH 6.4. The aqueous extract, after 30 minutes stirring was removed, concentrated *in vacuo* and dried by sublimation from the frozen state. Yield: 20.2 grams of brown powder. A second aqueous extract of the butanol yielded after similar treatment an additional 6.6 grams of brown powder.

Assay results were as follows:

Material	Response to diffusion plate assay No dilution.	1:4 dilution.
Starting broth	24.2 mm.	20.0 mm.
Clarified broth	25.2 mm.	20.4 mm.
Extracted broth:		
first 30 litres	14.8 mm.	none (no inhibition)
second 48 litres	10.4 mm.	„
Acid water wash	none	
First aqueous extract (10 litres)	30.0 mm.	28.2 mm.
Second aqueous extract	26.0 mm.	23.1 mm.
Solids from first extract at concentration of 1 mg/ml—This is solid $ 7	26.0 mm.	23.1 mm.
Solids from second extract at concentration of 1/mg/ml—This is solid $ 8	28.9 mm.	27.0 mm.

EXAMPLE 5.

In a later run, 775 gal., of unfiltered broth was adjusted to pH 2.3 and extracted with 310 gal. of *n*-butanol. The butanol extract was separated and washed with 40 gal. of water at pH 2, then extracted successively with 40 and 20 gal. portions of water, the mixture in each case being adjusted to pH 7.3—7.4 with sodium hydroxide. The aqueous extracts were combined and carried through a second acid butanol and alkaline water extracted series yielding a final rich aqueous extract of 12.1 litres which was spray-dried to give 420 grams of product.

EXAMPLE 6.

Carbon clarification and solvent precipitation of crude material. Twenty grams of the crude sodium salt of Amphomycin, prepared as described above in Example 5, was dissolved in 500 ml. of water, adjusted to pH 2 with phosphoric acid and extracted with 300 ml. of *n*-butanol to give a dark brown extract. The separated butanol layer was filtered through a diatomaceous filter aid to remove traces of undissolved water and stirred with 20 grams of activated charcoal ("Darco KB," "Darco" is a Registered Trade Mark) for 30 minutes. The carbon was removed by filtration and the light yellow butanol solution concentrated *in vacuo* to a volume of 100 ml. This concentrate was added to 2000 ml. of ethyl acetate, giving a copious precipitate of Amphomycin which was removed by filtration, washed with acetone, and dried. Yield: 3.4 grams of pale cream-coloured solids. Assays were as follows:

Material	Diffusion Plate Assay Responses No. dilution.	1:4 dilution.	1:16 dilution.
20 g. starting material at 1/mg/ml.	23.1 mm.	21.1 mm.	15.8 mm.
3.4 g. purified product at 1 mg/ml.	32 „	27 „	18 „

EXAMPLE 7.

ISOELECTRIC PRECIPITATION.

A 10 gram sample of Amphomycin prepared by butanol extraction and purification through the carbon treatment and ethyl acetate precipitation as described above was dissolved in 100 ml. of water, to give a clear solution at pH 2.2. The solution was adjusted to pH 3.4 with dilute sodium hydroxide, yielding a viscous, oily precipitate. The mother liquor was separated by decantation, adjusted to pH 6.6 with sodium hydroxide and lyophilised to yield 4.55 grams of solids (A). The precipitate was dissolved in water, adjusted to pH 6.8 and lyophilized, giving 5.68 grams of solids (B).

Material	Diffusion Plate Assay Response 1:4 dilution.	1:16 dilution.	1:64 dilution.
Starting material at 1 mg/ml. conc. - - -	29.0 mm.	23.7 mm.	14.7 mm.
Non-precipitated solids (A) - - - -	26.7 mm.	21.0 mm.	11.0 mm.
Precipitated solids (B) - - - - -	29.0 mm.	24.0 mm.	17.0 mm.

EXAMPLE 8.

PURIFICATION WITH REINECKE SALT.

Twenty-five grams of the sodium salt of Amphomycin was dissolved in 100 ml. of water and acidified with phosphoric acid to pH 2. This solution was extracted with 250 ml. of *n*-butanol. The wet butanol extract was treated with 25 grams of activated charcoal (Darco G—60) for one-half hour and filtered. The filtrate was extracted with 500 ml. of water at pH 7.0. The water layer was concentrated slightly *in vacuo* to remove butanol and adjusted to pH 1.9 with phosphoric acid. Twenty-five grams of Reinecke salt in 200 ml. of water was adjusted to pH 1.9 and added to Amphomycin solution. The resulting precipitate was removed by filtration after standing overnight in the refrigerator. The precipitate was redissolved in water by adjusting to pH 7.0 and reprecipitated by adjusting to pH 2.0. The reprecipitated material was removed by filtration, washed with water, dissolved in 150 ml. acetone and filtered. Addition of 1 litre acetone and concentrated ammonium hydroxide to pH 7.5 precipitated the ammonium salt of Amphomycin. The material was washed and dried. Yield 12.4 grams.

Diffusion plate Assays gave the following results:

Material	1:16 dilution.	1:64 dilution.	1:256 dilution.
Starting material at 1 mg/ml. concentration	25.3 mm.	20.7 mm.	16.0 mm.
Final product at 1 mg/ml. concentration - -	26.0 mm.	21.8 mm.	17.5 mm.

What we claim is:—

1. A process for producing Amphomycin which comprises cultivating a strain of Streptomyces canus or mutants thereof in an aqueous, nutrient containing carbohydrate solution under submerged aerobic conditions until substantial antibacterial activity is imparted to said solution and then recovering the so produced Amphomycin from the fermentation broth.

2. A process for producing Amphomycin which comprises cultivating a strain of Streptomyces canus or mutants thereof in an aqueous, nutrient containing, carbohydrate solution under submerged aerobic conditions at a temperature of from about 24° C. to about 30° C. for a period of from about two days to seven days, until substantial antibacterial activity is imparted to said solution and then recovering the so produced Amphomycin from the fermentation broth.

3. A process as claimed in claim 1, wherein the recovery of the Amphomycin includes the step of decolourisation of solutions of Amphomycin by activated charcoal.

4. A process as claimed in claim 1, wherein the recovery of the Amphomycin includes the step of extracting the antibiotic into a butyl alcohol or an amyl alcohol at a pH below 3.5.

5. A process as claimed in claim 1, wherein the recovery of the Amphomycin includes the step of precipitating the Amphomycin from aqueous solution by adjusting the pH to a point within the range of pH 3.0—4.0.

6. A process as claimed in claim 1, wherein the recovery of the Amphomycin includes the step of precipitating the Amphomycin from aqueous solution by adjusting the pH to a point within the range of pH 3.4—3.5.

7. A process as claimed in claim 1, wherein the recovery of the Amphomycin includes the step of removing impurities from an aqueous solution of Amphomycin having a pH below 3.5 by extraction of the impurities with methyl isobutyl ketone or amyl acetate.

8. A process as claimed in claim 1, wherein the recovery of Amphomycin includes the step of extracting the Amphomycin from a solution in butanol having a pH below 3.5 by the use of water having a pH higher than 4.

9. A process as claimed in claim 1, wherein the recovery of Amphomycin includes the step of precipitating the Amphomycin from solution by formation of insoluble derivatives of the basic function.

10. A process as claimed in claim 1, wherein the recovery of Amphomycin includes the step of precipitating the Amphomycin from solution by the formation of insoluble derivatives of the acidic function.

11. A process as claimed in claim 1, wherein the recovery of Amphomycin includes the step of extracting the antibiotic from solution in a water-immiscible organic solvent into water whose pH is greater than 6.0.

12. An antibiotic, Amphomycin, which is effective in inhibiting the growth of B. mycoides, B. aureus, M. tetragenus, Staph. aureus, and B. subtilis, is capable of forming salts with acids and metals, that is soluble in water, that exhibits minimum solubility in water between pH 3.0 and 4.0 that is readily soluble in methanol as the acid form and as the salt form, that is soluble in higher alcohols, containing at least four carbon atoms only in the acid form, that is extractable from water at pH 2 by butanol and pentanol, that is not extractable from water at pH 2 by methyl isobutyl ketone, benzene, ether, ethyl, acetate, and amyl acetate, that yields antibacterially active, solid derivates of ammonium hydroxide and of Reinecke's salt, that absorbs ultra-violet light only in the 210—230 mu region, that exhibits negative response to ninhydrin, Sakaguchi, Molisch and Ehrlich-Pauly tests, and that is stable for at least 10 days in aqueous solution from pH 2 to 10, and the acid and metal salts of said substances.

13. A process for producing an Amphomycin fermentation broth, which comprises cultivating a strain of Streptomyces canus or mutants thereof in an aqueous, nutrient-containing, carbohydrate solution under aerobic conditions, until substantial anti-bacterial activity is imparted to said solution.

14. A process for preparing Amphomycin substantially as described with reference to any one of the specific examples hereinbefore set forth.

15. Amphomycin when produced by the process particularly described herein.

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